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Integrinα5-Dependent Fibronectin Accumulation for Maintenance of Somite Boundaries in Zebrafish Embryos

Sumito Koshida,[1,2,3] Yasuyuki Kishimoto,[2,4] Hideko Ustumi,[1] Toshihiro Shimizu,[1] Makoto Furutani-Seiki,[2] Hisato Kondoh,[2,5] and Shinji Takada[1,2,3,*]

[1]Okazaki Institute for Integrative Bioscience
National Institutes of Natural Sciences
Okazaki 444-8787
Japan

[2]Exploratory Research for Advanced Technology
Kondoh Differentiation Signaling Project
Solution Oriented Research for Science and Technology Program
Kondoh Research Team
Japan Science and Technology Agency
Sakyo-ku, Kyoto 606-8305
Japan

[3]Department of Molecular Biomechanics
The Graduate University for Advanced Studies, SOKENDAI
Okazaki, Aichi 444-8787
Japan

[4]National Institute of Genetics
Mishima, Shizuoka 411-8540
Japan

[5]Graduate School of Frontier Biosciences
Osaka University
Suita 565-0871
Japan

Summary

Boundary formation and epithelialization are crucial processes in the morphological segmentation of vertebrate somites. By a genetic screening procedure with zebrafish, we identified two genes, *integrinα5* (*itga5*) and *fibronectin* (*fn*), required for these processes. Fibronectin proteins accumulate at somite boundaries in accordance with epithelialization of the somites. Both Fibronectin accumulation and the epithelialization are dependent on *itga5*, which is expressed in the most medial part of somites. Although somite boundaries are initially formed, but not maintained, in the anterior trunk of the mutant embryos deficient in either gene, their maintenance is defective at all axial levels of embryos deficient for both of these genes. Therefore, Integrinα5-directed assembly of Fibronectin appears critical for epithelialization and boundary maintenance of somites. Furthermore, with an additional deficiency in *ephrin-B2a*, the segmental defect in *itga5* or *fn* mutant embryos is expanded posteriorly, indicating that both Integrin-Fibronectin and Eph-Ephrin systems function cooperatively in maintaining somite boundaries.

*Correspondence: stakada@nibb.ac.jp

Introduction

Somites are metameric epithelial segments transiently formed along both sides of the embryonic body axis. Epithelial somites are generated repeatedly in an anterior to posterior (A-P) order by pinching off from the anterior end of the unsegmented mesenchymal precursor tissue, called the presomitic mesoderm (PSM). Prior to morphological segmentation, a segmental prepattern is established in the PSM (Holley and Takeda, 2002; Pourquie, 2003; Rida et al., 2004). This process is driven by a molecular oscillator, referred to as the segmentation clock, in which the Notch signaling pathway and various *hairy/Enhancer of split*-related (*Hes* in humans and mice and *her* in zebrafish) genes are involved. In contrast, the molecular mechanisms underlying morphological segmentation, including intersomitic boundary formation and somite epithelialization, are not yet clearly understood.

Molecular events occurring in the anterior PSM lead to morphological segmentation. In the mouse, the A-P polarization within a presumptive somite is established prior to the morphological segmentation by the Notch signaling and Mesp2, a bHLH transcription factor (Saga and Takeda, 2001; Takahashi et al., 2000). In addition, the T-box transcription factor *fused somites (fss)/tbx24*, which was identified by a large-scale screening of zebrafish, as well as Foxc winged helix transcription factors (Kume et al., 2001; Topczewska et al., 2001) also play essential roles in the formation of the proper A-P polarity in the anterior and/or intermediate PSM (Nikaido et al., 2002; van Eeden et al., 1996). Molecular interaction between cells with posterior identity and those with anterior identity at the interface between presumptive somites appears to result in morphological boundary formation, which involves mesenchymal-to-epithelial transition of cells at the boundaries. Modulation of the Notch activity appears to trigger boundary formation at the presumptive interface of somites (Sato et al., 2002). In addition, EphA4-Ephrin interaction at the presumptive interface of somites also plays a role in the morphological segmentation (Barrios et al., 2003; Durbin et al., 1998; Durbin et al., 2000).

Fibronectin may also be involved in the morphological segmentation. Fibronectin is a major component of the extracellular matrix (ECM) and involved in a variety aspects of cellular processes, including cell-substratum adhesion, cell migration, cytoskeletal organization, and cell proliferation. Fibronectin signaling is transmitted to the cytoplasmic components through Integrin receptors on the plasma membrane. During somitogenesis, Fibronectin, as well as focal adhesion proteins paxillin and focal adhesion kinase (FAK), which are activated by ECM-Integrin association and have a role in cell adhesion, accumulate at the somite boundary, suggesting that Fibronectin-Integrin association may function in the formation of the somite boundaries (Crawford et al., 2003; Henry et al., 2001). Actually, the gene *integrinα5* (*itga5*), which encodes a subunit of a Fibronectin receptor, is required for the epithelialization of

somites in mice (Goh et al., 1997). Mouse embryos deficient in *itga5* die at E9.5 to E10.5 with embryonic and extraembryonic defects, including arrest of posterior trunk elongation, absence of posterior somites, and vascular defects. Furthermore, *itga5*-null embryos show defective epithelialization of somites depending on their genetic background (Yang et al., 1999). Mouse embryos deficient in *fn* exhibit a similar but stronger phenotype than those deficient in *itga5* (George et al., 1993; Yang et al., 1999). For example, no distinctive somite formation is observed in *fn*-null embryos. However, although these studies show that Integrinα5 and Fibronectin are involved in various aspects of somitogenesis, including epithelialization of somites, the cellular basis of their functions is still unclear.

To gain insight into the mechanism underlying somite development, we performed an ENU mutagenesis screening of zebrafish and found that *integrinα5* and *fibronectin* were mutated in embryos showing defective boundary formation in their anterior somites; although no somite phenotype had been previously reported for *itga5* or *fn* mutations in zebrafish (Crump et al., 2004; Trinh and Stainier, 2004). Precise analysis with these mutants strongly suggests that Integrinα5-dependent accumulation of Fibronectin proteins is a critical event for the maintenance of somite boundaries and for the epithelialization of cells at the boundaries.

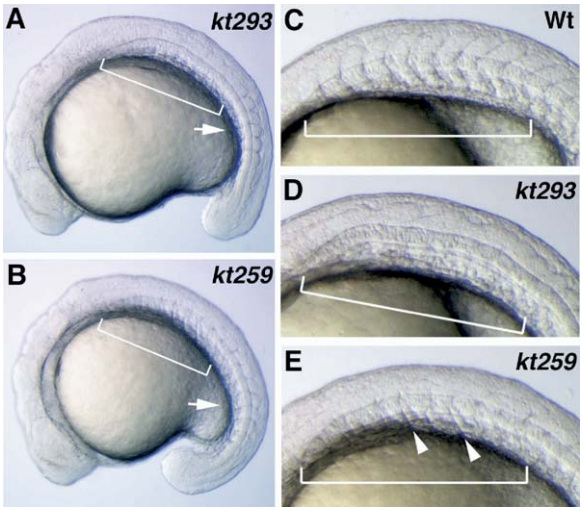

Figure 1. Isolation of Zebrafish Mutants Defective in Anterior Somite Development

(A and B) Morphological phenotypes of *kt293* (Group 1 [A]) and *kt259* (Group 2 [B]) embryos at the 16-somite stage. In these mutants, the anterior somites are fused (brackets), whereas the posterior somites form normally (arrows).

(C–E) Morphology of anterior somites of wild-type (C), *kt293* (D), and *kt259* (E) at the 18-somite stage. Arrowheads in (E) indicate the somite boundaries formed on the ventral side.

Results

integrinα5 and *natter/fibronectin* Are Genes Affected in Zebrafish Mutants Defective in Anterior Somite Development

To better understand the molecular mechanism underlying somite development, we performed ENU mutagenesis screening for mutations affecting morphogenesis of somites in zebrafish. In 615 F2 families, which corresponded to 662.2 mutagenized genomes, we obtained 15 mutants with defects in the formation of epithelial somites. Five of these mutants showed phenotypes similar to those of mutants previously identified, i.e., *fused somite*, *after eight*, *deadly seven*, *beamter*, and *mind bomb* (van Eeden et al., 1996), whereas the other ten showed distinctive phenotypes different from them. In five of them, the anterior two to ten somites were fused, whereas the posterior somites formed normally (Figure 1). Complementation analysis among these five mutations defined three complementation groups: Group 1, *kt293*, *kt451*, and *kt664* mutations; Group 2, *kt259*; and Group 3, *kt443* (see Table S1 in the Supplemental Data available with this article online). The Group 2 mutant, *kt259*, showed less severe somite defects than the mutants of Groups 1 and 3 because the ventral boundary of its somites often developed (Figures 1D and 1E). The phenotypes of the remaining five mutants were distinct and will be reported elsewhere.

To gain insight into the newly identified somite phenotypes, we first focused on Groups 1 and 2 and isolated the genes affected by these mutations. Mapping analysis with SSLP markers indicated that *kt293* (Group 1) was located close to z5141 (2/150 recombinants/meioses) on LG23, whereas *kt259* (Group 2) was located within the interval between z25375 (11/320) and z31778 (2/320) on LG9 (Figures 2A and 2E). Interestingly, *itga5* has been mapped to a site close to the region suspected to be mutated in *kt293*; and *fn*, which encodes a specific ligand for the Integrinα5β1 receptor, has been mapped to the region suspected to be mutated in *kt259* (Crump et al., 2004; Trinh and Stainier, 2004; Zhao et al., 2001). Thus, we tested *itga5* and *fn* as candidate genes for these mutations.

We found a polymorphism within an intron sequence of *itga5* (see Experimental Procedures) and detected no recombinant of this polymorphic marker with the *kt293* phenotype in 177 meioses (Figure 2A). Sequence analysis of *itga5* in *kt293* revealed a deletion of four nucleotides that cosegregated with the mutant phenotype. This mutation leads to a frameshift, resulting in synthesis a truncated version of the Integrinα5 protein. We also found a T to C nucleotide substitution in the splicing donor site of the fourth intron in the *kt451* allele that resulted in truncation before the third β-propeller repeat, an important region for ligand binding (Figure 2D) (Johansson et al., 1997; Springer, 1997). Because these two truncated forms of Integrinα5 lack the transmembrane domain at the carboxyl terminal region, we suppose that these two alleles are null. To test further whether dysfunction of Integrinα5 leads to the mutant phenotype, we carried out knockdown experiments with morpholino antisense oligonucleotides (MO). Injection of two individual MOs specific for *itga5* inhibited development of the anterior somite boundaries as observed in the Group 1 mutants (Figure 2B); whereas the injection of control MOs, carrying a 5-base substitution in each *itga5* MO, did not cause any significant effects (Figure 2C). Furthermore, injection of the wild-type *itga5* RNA at the 1-cell stage rescued the anterior so-

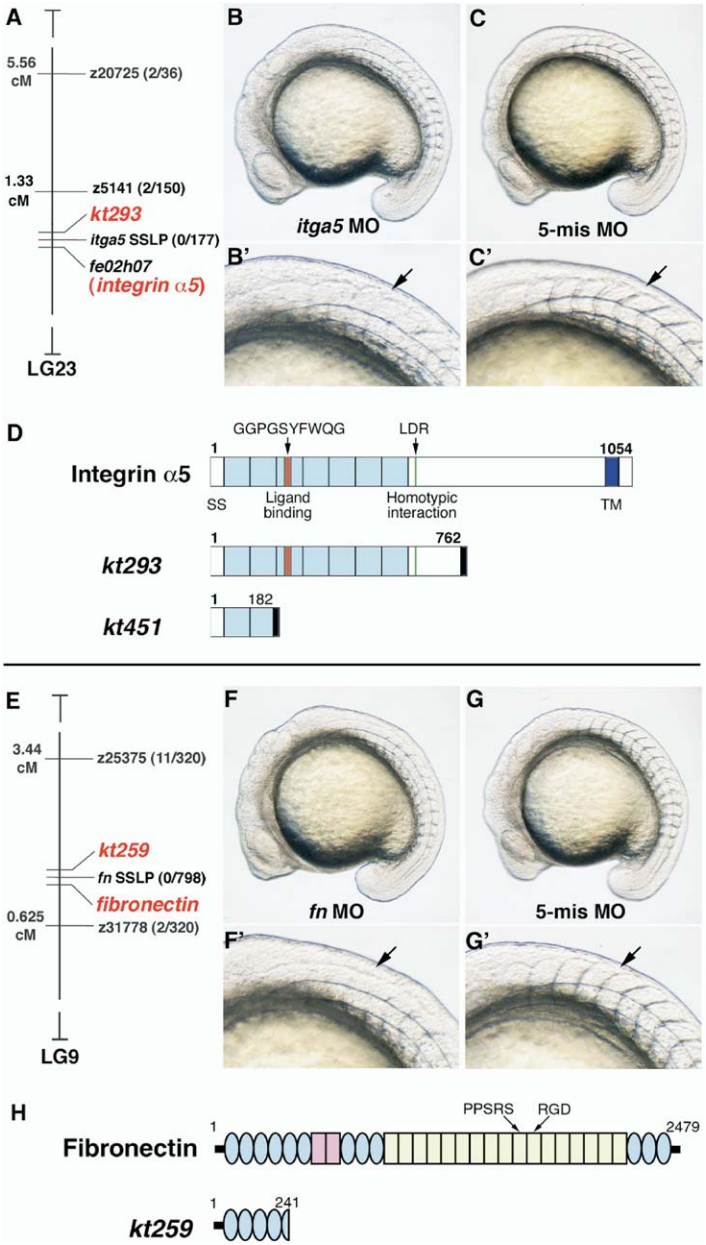

Figure 2. Mutated Genes of Group 1 and 2 Encode *integrinα5* and *fibronectin*, Respectively

(A and E) By use of SSLP markers, *kt293* (A) and *kt259* (E) were mapped to LG23 and LG9, respectively. Genetic distance from the mutation to each Z marker is denoted on the left side in cM.

(B, B′, C, and C′) Embryos injected with *itga5* MO (B, B′) and its control MO with a 5-base substitution (C, C′) at the 14-somite stage. *itga5* MO-injected embryos exhibit unclear boundaries between anterior somites. An arrow in B′ indicates a position where somite boundary appears unclear in an *itga5* MO-injected embryo, whereas an arrow in C' indicates the corresponding axial level to the arrow in B′.

(D) Schematic diagrams of Integrinα5 protein encoded by wild-type, *kt293*, and *kt451* alleles. Zebrafish Integrinα5 has a signal sequence (SS), 7 β-propeller repeats (light blue boxes), and a transmembrane domain (TM). Amino acid sequences demonstrated to be important for the ligand binding ability and the homotypic interaction are indicated.

(F, F′, G, and G′) Embryos injected with *fn* MO (F and F′) and its control MO with a 5-base substitution (G and G′) at the 14-somite stage. *fn* MO-injected embryos also exhibited unclear boundaries between anterior somites. As in the case of *itga5* MO-injected embryo, an arrow in F′ indicates a position where somite boundary appears unclear in an *fn* MO-injected embryo, whereas an arrow in G′ indicates the corresponding axial level to the arrow in F′.

(H) Schematic diagrams of Fibronectin protein encoded by wild-type and *kt259* allele. Type I (light blue ovals), type II (pink squares), and type III (yellow squares) repeats are shown. Amino acid sequences demonstrated to be the most important for Integrinα5β1 receptor recognition are indicated.

mite fusion phenotype of all alleles in this complementation group (see Table S2 in the Supplemental Data). Thus, we concluded the gene responsible for the somite phenotype in the Group 1 mutants to be zebrafish *integrinα5*.

We also identified a polymorphism within an intron sequence of *fn* (see Experimental Procedures) and detected no recombinant of this polymorphic marker with the *kt259* phenotype in 798 meioses (Figure 2E). Genomic sequence analysis of *fn* in *kt259* revealed an A to T substitution (AAG to TAG) that resulted in premature translational termination at codon 241 (Figure 2H). Because this truncated protein lacks almost all functional domains, including RGD and the synergy site, PPSRS, this *kt259* allele is likely null. As in the case of *itga5*, injection of two individual MOs specific for *fn*, but not control MOs, caused somites defect similar to those seen in the *kt259* mutant (Figures 2F and 2G). Recently, the gene responsible for the *natter* mutant phenotype, which shows defective myocardial migration, had been identified as *fn* (Trinh and Stainier, 2004). We confirmed that complementation crosses between *kt259* and the *tl43c natter* mutants produced embryos with defective anterior somite-boundary formation at the Mendelian frequency expected for noncomplementation of the *natter* mutation. Furthermore, as observed in *natter* mutants, the myocardial precursors were distributed into two separate populations away from the midline in *kt259* mutants at 24 hr postfertilization (hpf), whereas they fused at the midline to form a heart tube in normal siblings (data not shown). Therefore, we concluded that the new mutation, *kt259*, is allelic to the *natter* mutant.

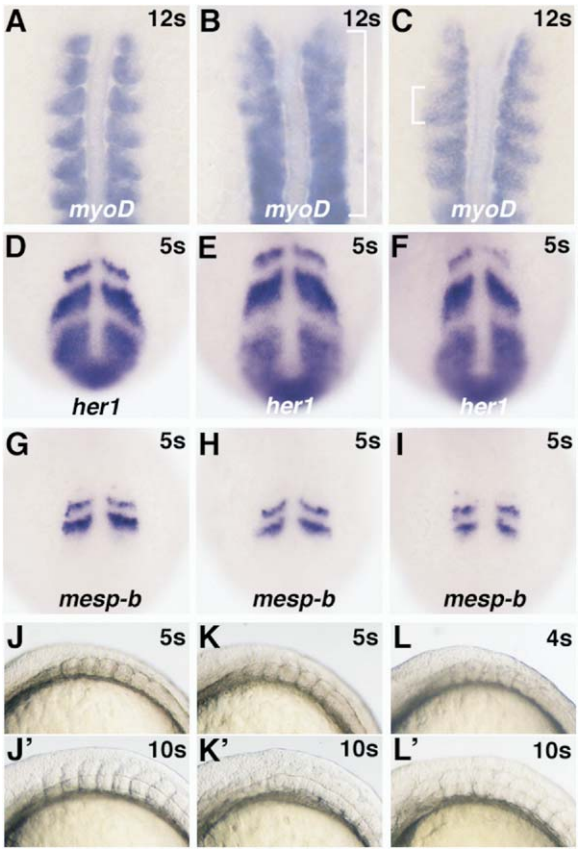

Figure 3. The Somite Boundary Is Generated Normally, But Not Maintained in *integrinα5* and *fibronectin* Mutants

(A–I) Expression patterns of *myoD* at the 10-somite stage, *her1* at the 5-somite stage, and *mesp-b* at the 5-somite stage in wild-type (A, D, G, respectively), *itga5* (B, E, H, respectively), and *fn* (C, F, I, respectively) mutant embryos. Anterior at the top. Although the expression domains of *myoD* in somites have fused in the mutants (brackets), *her1* and *mesp-b* are expressed normally in the PSM. (J–L and J′–L′) Morphological phenotypes of wild-type (J and J′), *itga5* (K and K′), and *fn* (L and L′) mutant embryos during early somitogenesis. Anterior at the left. Each pair of photos show the same embryo at the 5 (J–L)- and 10 (J′–L′)-somite stage. In the mutants, somite boundaries are seen at the 5-somite stage (K and L) but are not obvious at the10-somite stage (K′ and L′).

The Somite Boundary Is Generated Normally, But Not Maintained in *integrinα5* and *fibronectin* Mutants

To elucidate the function of Integrinα5 and Fibronectin in somitogenesis, we examined the expression patterns of genes involved in the segmentation process in the somites and PSM of *itga5* and *fn* mutants. The expression of *myoD* in the anterior somites was aberrant in the mutants; that is, the expression domains in the neighboring somites often fused (Figures 3A–3C). On the other hand, the expression of *her1* and *mesp-b*, which are considered to have important roles in the prepatterning of somite segmentation (Durbin et al., 2000; Henry et al., 2002; Holley et al., 2002; Oates and Ho, 2002; Sawada et al., 2000), were normal in the PSM of *itga5* and *fn* mutants (Figures 3D–3I). These observations suggest that the early segmentation process in the PSM proceeds normally in these mutants but that somite boundaries did not develop normally.

To examine the process of the defective boundary formation in *itga5* and *fn* mutants more precisely, we next observed the morphology of the anterior somites from early- to midsomite stages. At the 5-somite stage, somite boundaries were observed in both *itga5* and *fn* mutants; especially, the boundary between the newly formed somites and PSM was conspicuous (Figures 3J–3L). By the 10-somite stage, however, the boundaries had disappeared in the anterior somite areas in these mutants (Figures 3J′–3L′). In contrast, the boundaries between posterior somites were normal even in later stages. This transient boundary formation was also observed in embryos injected with *itga5* or *fn* MO (data not shown). These results indicate that morphologically distinct boundaries are initially generated, but not maintained, in the anterior somites in both *itga5* and *fn* mutant embryos.

integrinα5 and *fibronectin* Are Expressed in the Adaxial Cells and the Tailbud, Respectively

To better understand where and how Integrinα5 and Fibronectin function to maintain somite boundaries, we next examined the expression patterns of *itga5* and *fn* by in situ hybridization (Figure 4). At the 1-somite stage, *itga5* expression was seen in the PSM (Figures 4A and 4B) and at the anterior edge of the neural plate (Figure 4C) (Crump et al., 2004). At the 8-somite stage, *itga5* was expressed in the medial part of somites in a segmental manner (Figure 4D) as well as in the PSM, the anterior neural tissue, and mesenchymal cells around the otic vesicles (Figures 4E and 4F) (Crump et al., 2004). Two-color staining for *myoD* and *itga5* expression at the 12-somite stage revealed that *itga5* was segmentally expressed in the adaxial cells overlappingly with *myoD* (arrowheads in Figure 4G). On the other hand, *fn* was expressed strongly in the notochord and weakly in the PSM at the 1-somite stage (Figure 4I). As somitogenesis proceeded, *fn* expression was reduced in the notochord and became stronger in the posterior PSM (Figures 4J–4L).

Previous studies showed that Notch signaling components and the Fss/Tbx24 transcription factor play important roles in somite-boundary formation in the PSM (Holley et al., 2000; Holley et al., 2002; Nikaido et al., 2002). We thus examined the expression patterns of *itga5* and *fn* in *after eight (aei)/deltaD* mutant embryos and in *fss* mutant embryos (Figures 4M–4T). *itga5*-expressing domains in the adaxial cell region were fused in *aei* and in *fss* mutants (Figures 4N and 4R), whereas *fn* expression was not altered (Figures 4P and 4T). These results indicate that Notch signaling and Fss/Tbx24 activity in the PSM regulate the segmental expression pattern of *itga5*.

Accumulation of Fibronectin Protein at Somite Boundaries Is Dependent on *integrinα5*

Because Fibronectin is secreted into the extracellular space, in situ hybridization analysis does not provide precise information about the spatial distribution of this protein during somitogenesis. Thus, we carried out an immunostaining experiment with anti-Fibronectin poly-

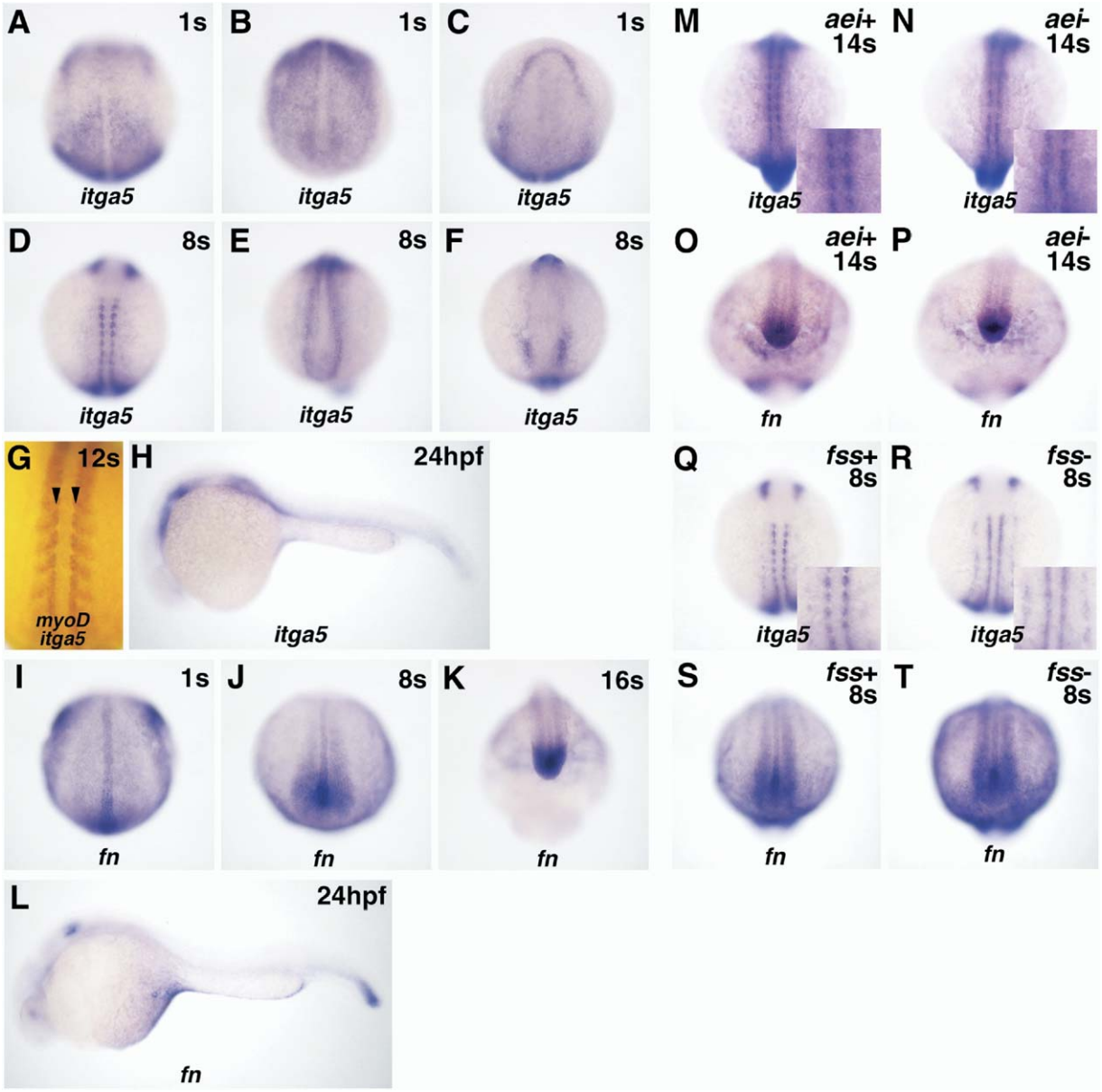

Figure 4. Expression Patterns of *integrin*α*5* and *fibronectin*

(A–H) Expression of *itga5* in wild-type embryos at the 1-somite stage (A–C), 8-somite stage (D–F), and at 24 hpf (H). Dorsal (A, D, and G), vegetal (B and E), animal (C and F), and lateral (H) views are shown. *itga5* is expressed in the adaxial cells and the PSM during somitogenesis. Arrowheads indicate the overlapped expression of *itga5* and *myoD*. Two-color in situ hybridization with *itga5* (purple) and *myoD* (red) probes at the 12-somite stage (G). *itga5* is expressed at the anterior edge of the neural plate and around the otic vesicles (C and F). Expression of *itga5* in the somites is reduced at 24 hpf (H).

(I–L) Expression of *fn* in wild-type embryos at the 1 (I)-, 8 (J)-, 16 (K)-somite stages and at 24 hpf (L). Dorsoposterior (I), posterior (J and K), and lateral (L) views. During somitogenesis, *fn* is expressed in the tailbud (I–K). At 24 hpf, *fn* is expressed the tailbud and the otic vesicles (L).

(M–T) Expression of *itga5* (M, N, Q, and R) and *fn* (O, P, S, and T) in *aei* (M–P) and *fss* embryos (Q–T) at the 14- and 8-somite stage, respectively. The insets in (M) and (N) and in (Q) and (R) provide higher magnification views. *itga5*-expressing domains in the adaxial cell region are fused in *aei* and *fss* mutants, whereas *fn* expression in the tailbud has not changed.

clonal antibody to gain further information about the place where Fibronectin functions. As previously reported (Crawford et al., 2003), Fibronectin protein was detected at somite boundaries in wild-type embryos (Figures 5A and 5B). The deposition of Fibronectin protein was prominent anterior to border 1 (B1) between SI (the newly segmented somite) and SII but was less obvious at B0, the boundary between SI and the PSM (Figures 5C and 5D) (Pourquie and Tam, 2001). Thus, the protein accumulated gradually at somite boundaries after they had once formed. This Fibronectin accumulation appears to be consistent with the pheno-

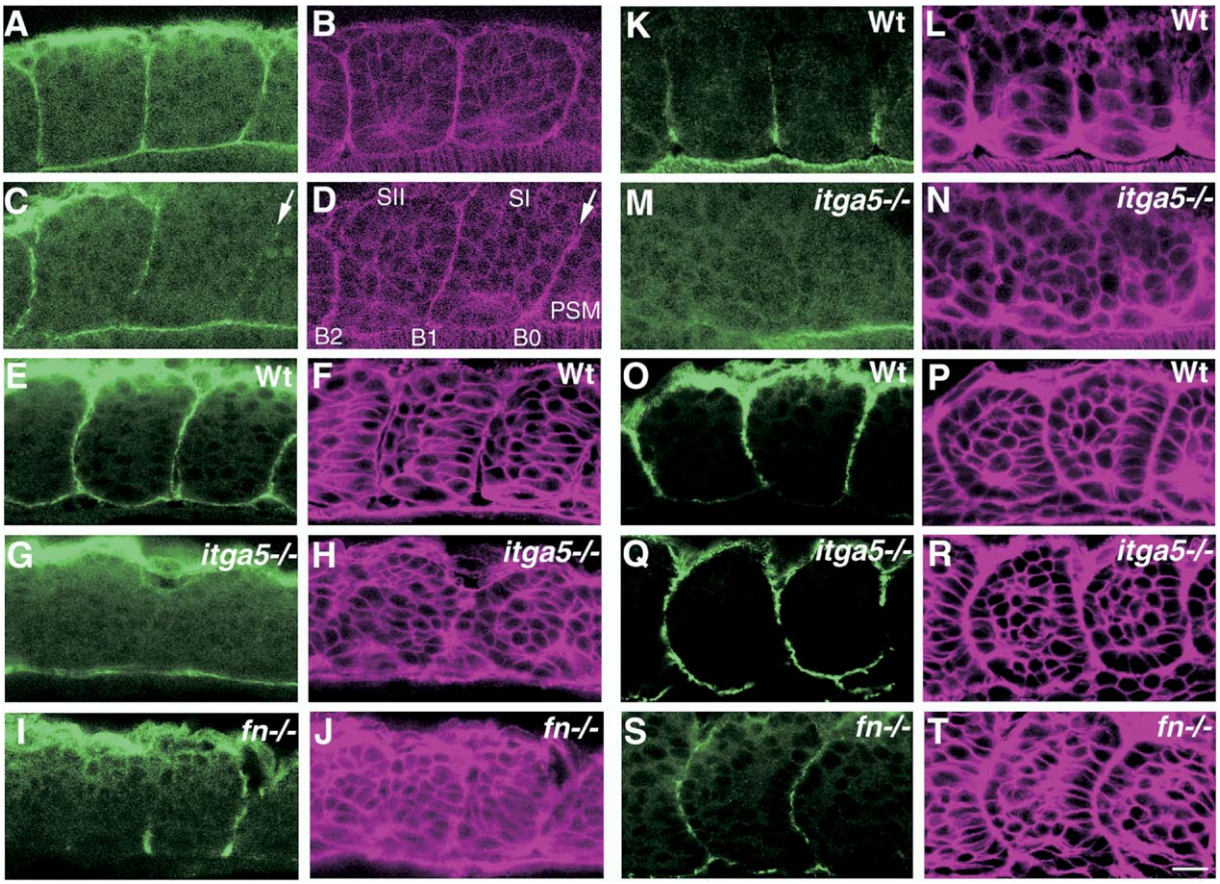

Figure 5. Fibronectin Protein Accumulation at Somite Boundaries Requires *integrin*α*5*

(A–D) Horizontal sections of the anterior somites (A and B) and the newly formed somites (C and D) stained with anti-Fibronectin antibody (A and C) and phalloidin (B and D) in wild-type embryos at the 9-somite stage. Anterior at the left. The Fibronectin signal is localized at somite boundaries. Arrows in (C) and (D) indicate the newly formed boundary. Fibronectin accumulation is not obvious. Nonspecific staining is observed in the epidermis.

(E–J) Parasagittal sections of the anterior somites in wild-type (E and F), *itga5* mutant (G and H), and *fn* mutant embryos (I and J) stained with anti-Fibronectin antibody (E, G, and I) and phalloidin (F, H, and J) at the 9-somite stage. Fibronectin accumulation at somite boundaries is diminished in the mutants.

(K–N) Horizontal sections of the anterior somites in wild-type (K and L) and *itga5* mutant (M and N) stained with anti-FAK[pY397] phosphospecific antibody (K and M) and phalloidin (L and N) at the 10-somite stage.

(O–T) Parasagittal sections of the posterior somites in wild-type (O and P), *itga5* mutant (Q and R), and *fn* mutant embryos (S and T) stained with anti-Fibronectin antibody (O, Q, and S) and phalloidin (P, R, and T) at the 16-somite stage. The scale bar represents 20 μm.

type of *fn* mutants because *fn* is required, not for generation of the somite furrow occurring at B0 but for its maintenance anterior to this position.

In embryos homozygous for *fn/nat*^{kt259}, the Fibronectin accumulation at somite boundaries was diminished in the anterior trunk (Figure 5I). Unexpectedly, the accumulation was not observed at somite boundaries in the anterior trunk of *itga5* homozygous mutant embryos (Figure 5G), although *fn* mRNA was normally expressed (data not shown). These results suggest that the accumulation of Fibronectin at the boundary of somites is important for the maintenance of the boundaries and that *itga5,* expressed in the adaxial cells, is required for this accumulation. In accordance with Fibronectin accumulation, FAK, which is a downstream component activated by Fibronectin, is accumulated at somite boundary and its phosphorylation level is increased (Crawford et al., 2003; Henry et al., 2001). In *itga5* homozygous mutant embryos, the accumulation of phosphorylated FAK was not detected at the boundaries between anterior somites (Figure 5M), suggesting that the activation of Fibronectin-dependent signaling was actually defective at somite boundaries in accordance with the defective accumulation of Fibronectin.

In contrast to its defective accumulation in anterior somites, Fibronectin accumulated normally at the somite boundaries in the posterior region of *itga5* homozygous mutant embryos, where the boundaries formed normally between the somites (Figure 5Q). In addition, binding of anti-Fibronectin antibody was also detected at the somite boundaries in the posterior region of *fn* homozygous mutants (Figure 5S). These results indicate that the accumulation of Fibronectin at the somite boundaries is closely correlated with the boundary for-

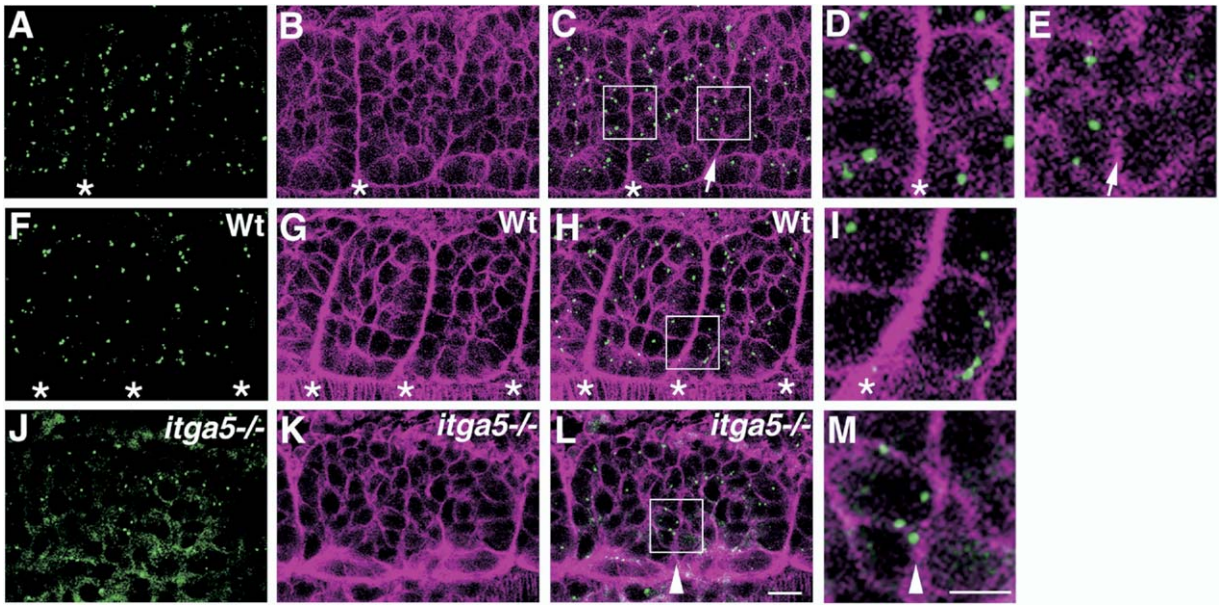

Figure 6. Cell Polarity in Somites Is Aberrant in *integrinα5* Mutants

Horizontal sections of the newly formed somite in a wild-type embryo (A–E) and of the anterior somites in wild-type (F–I) and *itga5* mutant embryos (J–M) stained with anti-γ-Tubulin antibody (A, F, and J) and phalloidin (B, G, and K) at the 9-somite stage. High-magnification views of the boxed area in (C), (H), and (L) are shown in the right panels (D, E, I, and M, respectively). The scale bars represent 20 μm (L) and 10 μm (M), respectively. Anterior at the left. Arrows and stars in (C) and (E) indicate the newly formed boundary and the other somite boundaries, respectively. Stars in (A)–(I) indicate somite boundaries. In wild-type embryos, centrosomes are localized apically (F–I), whereas centrosomes in the mutant cells formerly adjacent to the boundary (arrowheads in [L]) are positioned randomly (L and M).

mation. Furthermore, because both *itga5* and *fn* alleles used in this study appear to be null, these results suggest that some other genes compensate for the loss of function of Integrinα5 or Fibronectin in the posterior somites. Especially, positive immunostaining with the anti-Fibronectin antibody in the *fn* mutants suggests that proteins closely related to Fibronectin in structure function in the formation of posterior somite boundaries.

Epithelialization of Somites Is Aberrant in *integrinα5* and *fibronectin* Mutants

During somite formation, the cells adjacent to the somite boundary acquire epithelial cell characteristics, i.e., a columnar shape and apical localization of the centrosome (Barrios et al., 2003). To investigate whether the accumulation of Fibronectin at somite boundaries is involved in this mesenchymal-to-epithelial transition, we examined the morphology and the centrosome position in the cells located at somite boundaries in *itga5* and *fn* mutant embryos (Figure 6). In wild-type embryos, the columnar cell shape and polarized centrosome positioning were observed at levels anterior to B1, but not at B0 (Figures 6A–6E). In contrast, in *itga5* or *fn* mutant embryos, the cells formerly located at the boundaries between anterior somites were round and had centrosomes that were randomly positioned in their cytoplasm (Figures 6F–6M and data not shown), whereas the cell shape and centrosome positioning appeared normal at those between posterior somites, where the accumulation of Fibronectin was observed (data not shown). Thus, the accumulation of Fibronectin at the somite boundaries appears to be required for the epithelialization of the somite-boundary cells, which assures the maintenance of somite boundaries.

integrinα5 and *fibronectin* Are Also Required for the Maintenance of Posterior Somite Boundaries

In embryos deficient for *itga5* or *fn*, the approximately ten anteriormost somites eventually fused, whereas the boundaries between posterior somites were maintained. *itga5* and *fn,* however, are expressed throughout the A-P length of the somites during normal development, suggesting that *itga5* and *fn* may have roles even in posterior somite development. Injection of both MOs specific for *itga5* and *fn* caused loss of somite boundaries even in the posterior trunk in addition to the anterior one (Figures 7A–7C). Furthermore, as in the case of the anterior region, the boundaries were initially formed between newly formed somites in the posterior region but then fused as they mature in later stages (data not shown). Therefore, *itga5* and *fn* are involved in the maintenance of somite boundaries not only in the anterior but also in the posterior trunk.

Integrin-Fibronectin and Eph-Ephrin Systems Function Cooperatively in the Maintenance of Somite Boundaries

In addition to the Integrin-Fibronectin system, Eph-Ephrin signaling has been proposed to be involved in the morphological segmentation of somites (Barrios et

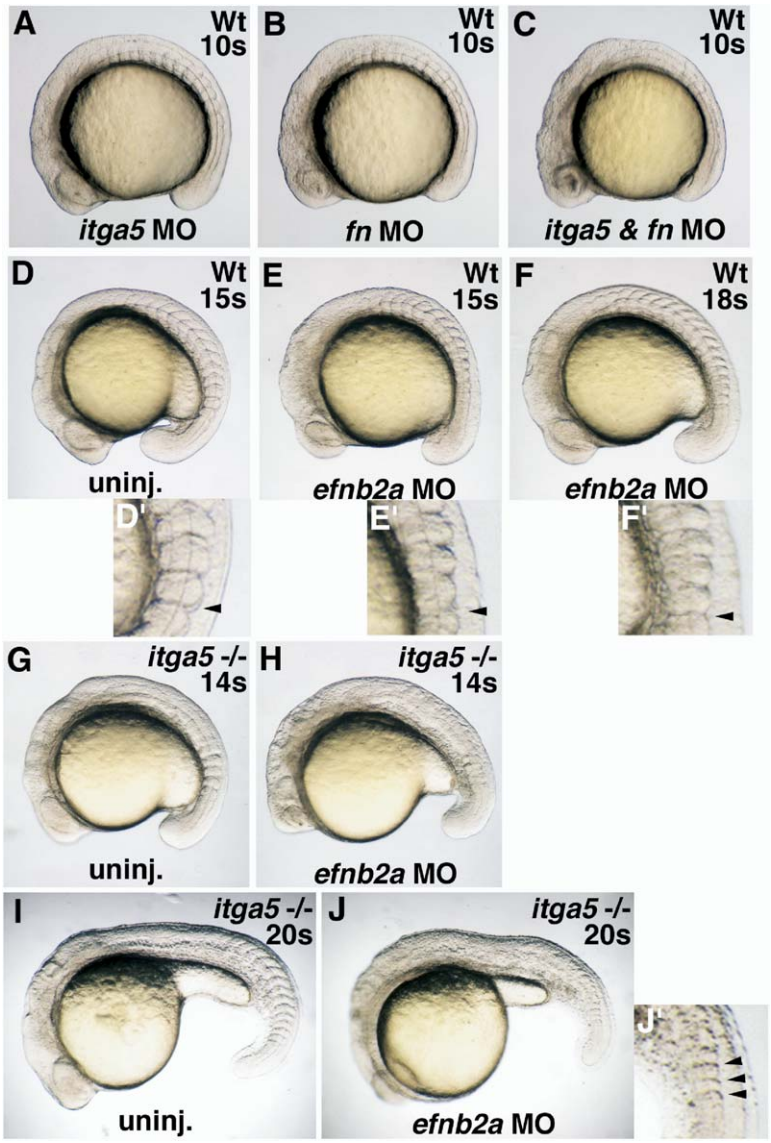

Figure 7. Integrin-Fibronectin System and Ephrin-B2a Function Redundantly during Posterior Somite Development

(A–C) *itga5* MO (A)-, *fn* MO (B)-, and *itga5* and *fn* MO (C)-injected wild-type embryos at the 10-somite stage. *itga5* and *fn* double morphants show much more severe defects in somites than either single morphants.

(D–F) Noninjected (D) and *efnb2a* MO-injected (E and F) wild-type embryos at the 15 (D and E)- and 18 (F)-somite stage. High-magnification views of the newly formed somites in (D)–(F) are shown in the lower panels ([D′]–[F′], respectively). Arrowheads indicate the anteriormost edge of the PSM. In *efnb2a* MO-injected embryos, the newly formed approximately four somite boundaries on the ventral side are unclear at the 15-somite stage (E and E′) but become obvious at the 18-somite stage (F and F′).

(G–J) Noninjected (G and I) and *efnb2a* MO-injected (H, J, and J′) *itga5* mutant embryos at the (G and H) 14- and (I, J, and J′) 20-somite stage. *efnb2a* MO-injected *itga5* mutant embryos show much more severe defects in somites than either *efnb2a* MO-injected or *itga5* mutant embryos. Distinct boundaries are initially formed even between posterior somites (arrowheads in [J′]).

al., 2003). To assess the correlation between these two systems, we examined whether genes involved in these systems were genetically interactive in the boundary formation between somites. We examined the phenotype of embryos deficient for both *itga5* and *ephrin-B2a* (*efnb2a*), one of the *ephA4* ligands expressed segmentally in the paraxial mesoderm. First, wild-type embryos injected with *efnb2a* MO showed a delay in somite-boundary formation on the ventral side during early somitogenesis (Figures 7D and 7E), although complete somite boundaries were eventually established by the 18-somite stage (Figure 7F). In contrast, *efnb2a* MO injection into *itga5* mutant embryos caused severe defects in somite-boundary formation: that is, almost all somites fused (Figures 7H and 7J). In these MO-injected embryos, distinct boundaries were initially formed between newly formed somites at the posterior end (Figure 7J′) but were not maintained in later stages, indicating that both Integrin-Fibronectin and Eph-Ephrin systems are required for maintenance of somite boundaries in the posterior region. The molecular characteristics of the posterior segmental process in these embryos appeared to be identical to those observed for the anterior segmental process in *itga5* or *fn* mutants; i.e., at the 18-somite stage, the expression of *myoD* was fused in both anterior and posterior somites of the MO-injected *itga5* mutants, whereas that of *her1* and *mesp-b* was normal (data not shown). A similar phenotype was also obtained by injecting *efnb2a* MO into *fn* mutant embryos (data not shown). These results strongly support the model that the Integrinα5-Fibronectin system functions in the maintenance of somite boundary in cooperation with the Eph-Ephrin system.

Discussion

Dissection of Morphological Processes Involved in Somite Segmentation

The formation of the somite boundary is an important process in the development of the metameric struc-

tures of vertebrate embryos. Although genetic mechanisms involved in generating the segmental pattern in the PSM has been extensively studied, the cellular basis of the morphological segmentation has remained elusive. As a result of this study, we propose that the process of somite-boundary formation can be subdivided into two steps: (1) initial establishment and (2) maintenance of the boundary.

We showed that Integrinα5 and its ligand, Fibronectin, play important roles in morphological segmentation of somites in zebrafish embryos. Fibronectin deposition was minimal at the newly formed boundary (B0) between the first newly formed somite (SI) and the anteriormost edge of the PSM, but Fibronectin progressively accumulated at the boundaries as the somites mature (Figure 5). Thus, Fibronectin accumulates at the somite boundary after the morphological boundary has been generated. Interestingly, this accumulation was disrupted not only in the *fn* mutant but also in the *itga5* mutant embryos in the anterior trunk, indicating that Fibronectin accumulated at somite boundaries in Integrinα5-dependent fashion. Furthermore, in the anterior regions of these mutants, somite boundaries were initially formed, but not maintained (Figure 3). Thus, Integrinα5-dependent Fibronectin accumulation at somite boundaries is likely to be essential for maintenance, but not for initial establishment of the formation of morphological boundary between somites. In other words, the morphological process of somite-boundary formation can be subdivided into the maintenance step, in which Fibronectin accumulation appears to be required, and the initiation step, which proceeds without this accumulation.

Mechanism of Fibronectin Accumulation at the Boundaries of Somites

Our results allow us to propose a model for the mechanism underlying the accumulation of Fibronectin, which is essential for the maintenance of the somite boundary. This accumulation was disrupted in the anterior trunk of zebrafish embryos deficient in *itga5* or *fn* (Figure 5). Thus, the Fibronection accumulation is dependent on Integrinα5. Interestingly, *itga5* was not expressed in cells at the boundary but rather in the adaxial cells, which exist in the most medial part of somites (Figure 4). Therefore, Integrinα5 proteins on the plasma membrane of the adaxial cells are likely required for the accumulation of Fibronectin at the somite boundaries. One possible mechanism to explain this Integrin-dependent accumulation of Fibronectin is the so-called Fibronectin fibrillogenesis (Schwarzbauer and Sechler, 1999). In this case, the binding of Fibronectin to its receptor, Integrinα5β1, may trigger off sequential intermolecular bindings between Fibronectin proteins, leading to the formation of a Fibronectin matrix. Thus, Integrinα5 may function as an indispensable anchor to form a laterally elongated Fibronectin matrix along the initially generated intersomitic furrows. An ectopic grafting of the adaxial cells or Integrinα5-expressing culture cells into paraxial mesoderm may reveal whether Integrinα5 can trigger the assembly of Fibronectin.

Roles of Fibronectin in the Epithelialization of Boundary Cells

Concomitant with segmentation boundary formation, epithelialization takes place in cells at the boundary. These cells acquire a columnar morphology accompanied by apical relocalization of their centrosome (Barrios et al., 2003). In wild-type embryos, the acquisition of a columnar shape and the establishment of cell polarity were not yet observed at the newly formed boundary (B0) but were found in boundary cells anterior to B1, in accordance with the Fibronectin accumulation (Figures 5C and 5D and 6A–6E). This observation suggests that accumulated Fibronectin at the boundary promoted epithelialization. In fact, in the anterior trunk of *itga5* or *fn* mutant embryos, in which the Fibronectin accumulation was defective, the boundary cells were round and exhibited a random positioning of the centrosome within their cytoplasm.

Little is known about the molecular mechanism accounting for the accumulated Fibronectin leading to further epithelialization. No receptor that binds to Fibronectin and transmits this signal to an intracellular signaling pathway has yet been identified in cells at the somite boundary. Localization and activation of Integrin signaling components, however, supports the idea that Integrin-mediated cell-matrix interaction is involved in the somite development. For instance, focal adhesion kinase (FAK) and paxillin, which are recruited to the Integrin receptor when this receptor binds to matrix and associates with the actin cytoskeleton, have been shown to be concentrated at somite boundaries (Crawford et al., 2003; Henry et al., 2001). At these boundaries, FAK is phosphorylated on Tyr397, indicating that the Integrin signaling is activated. In contrast, we showed that this phosphorylated form of FAK was completely abolished in *itga5* mutants (Figures 5K–5N). This result strongly suggests that Integrin-mediated cytoskeletal reorganization is involved in the process of epithelialization induced by Fibronectin.

Other components that might be involved in this process are Rho family GTPases Cdc42 and Rac1, known to be major regulators of cytoskeletal rearrangement (DeMali et al., 2003). In chick embryos, Cdc42 plays a role in the binary decision of somite cells between epithelial and mesenchymal states. On the other hand, the proper level of Rac1 is required for somite epithelialization (Nakaya et al., 2004). At present, it is not certain whether these two Rho family GTPases are involved in the Integrin-Fibronectin-dependent process or in some other process in somite epithelialization. Further studies to address this question are awaited.

Difference in Segmental Defects between Anterior and Posterior Somites in *itga5* or *fn* Mutant Embryos

In contrast to those in the anterior region, the somite boundaries in the posterior region clearly formed in *itga5* or *fn* mutant embryos. In these mutants, anti-Fibronectin antibody bound along somite boundaries in the posterior region of either *itga5* or *fn* mutant embryo (Figures 5O–5T). This accumulation of immunoreactivity toward anti-Fibronectin antibody may explain the reason why the somite-boundary formation proceeded in the posterior region of *itga5* or *fn* mutant embryos. Further-

more, this observation also provides more insight into the mechanism of the somite-boundary formation. Because the *itga5* mutant alleles used in this study are likely to be null, this observation may imply that the somite-boundary formation and the assembly of Fibronectin can proceed without Integrinα5. Furthermore, because the truncated proteins produced by the *fn* mutant allele are not likely to assemble each other, the accumulated immunoreactivity at the posterior intersomitic boundaries suggests that another molecule immunoreactive with anti-Fibronectin antibody accumulates at somite boundaries in this region. Because a *fibronectin* related zebrafish gene has been identified, this *fn* related gene may function in the posterior region. Together, some genes encoding Integrinα5- or Fibronectin-related proteins may function in the posterior region in place of Integrinα5 or Fibronectin.

If the molecular machinery that compensates for the loss of *itga5* or *fn* functions in the posterior region, a correlation between this machinery and Integrinα5-Fibronectin system should be revealed. Interestingly, in embryos deficient for both *itga5* and *fn*, the boundary formation was defective in both posterior and anterior regions. In this embryo, almost all boundaries were absent along the entire body axis, and only a few boundaries formed between the most posterior somites throughout somite-forming stages. Because these boundaries were diminished in later stages, *itga5* and *fn* are also required for the maintenance of somite boundaries even in the posterior region. Thus, *itga5* and *fn* function in the maintenance of the somite boundary along the entire body axis; either *itga5* or *fn* is required for the boundary maintenance in the posterior region, whereas both genes are required in the anterior region. Thus, the total amount of Integrin and Fibronectin proteins may be critical for the maintenance of the somite boundary, and the required amount may be different for the process between the anterior and the posterior regions.

Roles of Eph-Ephrin System in Morphological Segmentation

Eph-Ephrin signaling has been implicated in the boundary formation of somites and epithelialization of cells at the boundary. The expression of *ephA4* is restricted to one or two rows of cells in the most anterior region of the presumptive somites as well as to those cells in the newly formed somite. In contrast, *ephrin-B2a* and *ephrin-A1*, which encode ligands for the EphA4 receptor, show graded expression within the presumptive somites as well as in the somites with the highest expression being in posterior cells adjacent to the forming boundary. Disruption of Eph-Ephrin signaling by injection of RNA encoding dominant-negative forms of Eph and Ephrins results in defects in boundary formation of somites (Durbin et al., 1998). Furthermore, restoration of EphA4-Ephrin signaling in *fss* mutant cells, in which EphA4 expression is lost and somite boundary and epithelial somites are not formed, results in the formation of morphological boundaries and the epithelialization of EphA4-expressing cells (Barrios et al., 2003; Durbin et al., 2000). Thus, Eph-Ephrin signaling appears to play roles in most aspects of boundary formation of somites and epithelialization of cells at the boundary. Under our study conditions, we found that the Eph-Ephrin system may function redundantly with Integrin-Fibronectin system in the boundary formation between posterior somites; because little boundary formation was observed in *itga5* or *fn* mutant embryos defective in *efnb2a*, whereas the posterior boundary formation appeared normal in embryos defective in either *itga5* or *fn*, or only slightly delayed in those defective in *efnb2a* (Figures 7D–7J). Furthermore, even with an additional deficiency in *ephrin-B2a*, the initial establishment of somite boundaries occurred normally in *itga5* mutant embryos, suggesting that Ephrin-B2a also functions in the maintenance of somite boundaries (Figure 7J′). Thus, in our present study, the function of the Eph-Ephrin system in the maintenance of the boundary was highlighted, and this function appears to parallel that of the Integrin-Fibronectin system. Because *efnb2a* is expressed in the most posterior region of somites, some unidentified Eph molecule that is expressed in the anterior region of somites may participate as an Ephrin-B2a receptor in this process. Our study indicates that the morphological segmentation of vertebrate somites can be divided into two processes: boundary establishment, which does not require *itga5* and *fn*, and its maintenance, which is dependent on these genes as well as *efnb2a*. To understand the mechanism underlying the somite-boundary formation and the epithelialization of cells at the boundaries, we also need to identify the other molecules involved in these processes and to examine the interaction between such molecules and the Integrin-Fibronectin system.

Experimental Procedures

Fish Strains and Mutant Screening

All studies on wild-type zebrafish (*Danio rerio*) were carried out using fish having the TL2 closed colony background (Kishimoto et al., 2004). Three previously identified mutant strains, *fused somites*^{ti1}, *after eight*^{tr233}, and *natter*^{tl43c}, were also used in this study. Embryos obtained from natural crosses were maintained in egg water (0.03% artificial sea salt in reversed osmosis water) at 23°C or 28°C.

ENU-based mutagenesis was performed as described previously (Kishimoto et al., 2004). F3 embryos were screened at the 15- to 20-somite stage by morphological observation under a stereomicroscope.

Genetic Mapping

kt293 and *kt259* heterozygous fish were mated with wild-type Tuebingen fish to generate F1 families. Homozygous mutant embryos were obtained from the F1 crosses. Then we performed PCR reactions for specific SSLP markers by using their genomic DNA. The PCR was performed under the following conditions: 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min (30 cycles). To map the mutations, we designed SSLP markers within intron regions of *integrinα5* and *fibronectin* (*itga5* SSLP primers, 5′-TATATAGAAATT GAAATAGC-3′ and 5′-AACCGTGATTAATTGCATCC-3′; *fn* SSLP primers, 5′-CTGTCTGTTTTTAATCTAGG-3′ and 5′-ATTGTTGGTAG TTTTCCCTG-3′).

Injection of Morpholino Oligonucleotides and RNA

Morpholinos (GeneTools) and a capped RNA synthesized by using an mMESSAGE mMACHINE (Ambion) were injected into 1-cell-stage embryos at a concentration of 2–3 mg/ml (*itga5* MO1, 5′-CATAGTAACCGATGTATCAAAATCC-3′; *itga5*-5mis MO1, 5′-CATAc TAACgGATcTATgAAAAaCC-3′; *itga5* MO2, 5′-ACTGCTTTATTAAA CTTCTTTCACA-3′; *itga5*-5mis MO2, 5′-ACTcCTaTATTAAAgTTa TTTaACA-3′; *fn* MO1, 5′-CCGTGCCCAAAGGGCCACCAAACAT-3′;

fn-5mis MO1, 5′-CgGTGCCgAAAGcGCCAgCAAAgAT-3′; *fn* MO2, 5′-CACAGGTGCGATTGAACACGCTAAA-3′; *fn*-5mis MO2, 5′-CAg AGcTGCGATTcAAgACGgTAAA-3′), 5 mg/ml (*efnb2a* MO, 5′-AAT ATCTCCACAAAGAGTCGCCCAT-3′), or 0.1 mg/ml (*itga5* RNA) in distilled water.

In Situ Hybridization and Immunohistochemistry

In situ hybridization experiments were performed as described previously (Nikaido et al., 1997). For immunostaining, embryos were fixed in 4% PFA/PBS for 1 hr at room temperature. For detection of Fibronectin and pFAK[Y397], embryos were permeabilized with 0.5% Triton X-100 in PBS at room temperature for 1.5 hr, rinsed with PBS, and then incubated in 2% BSA, 10% DMSO, and 0.2% Triton X-100 in PBS. For γ-Tubulin, embryos were permeabilized by acetone treatment at −20°C for 5 min, washed in PBST (0.1% Triton X-100 in PBS), and then incubated in blocking solution (5% serum in PBST). Polyclonal anti-fibronectin antibody (Fibronectin Ab-10, NeoMarkers), polyclonal anti-FAK [pY397] phosphospecific antibody (BioSource International), and monoclonal anti-γ-Tubulin antibody (GTU-88, Sigma) were used at 1:500, 1:200, and 1:1000 dilutions, respectively. Alexa 488 goat anti-rabbit IgG and Alexa 488 goat anti-mouse IgG (Molecular Probes) were used as secondary antibodies at a 1:250 dilution. Rhodamine-phalloidin (Molecular Probes) was added at a 1:200 dilution to the solution containing a secondary antibody to detect F-actin. Embryos were washed with PBSDT (1% DMSO and 0.1% Triton X-100 in PBS; for Fibronectin and FAK[pY397]) or with PBST (for γ-Tubulin) at room temperature after incubation with each antibody. The specimens were embedded in 5% agar and cut into 50-μm sections with a microslicer and then observed by LSM510 confocal laser scanning microscopy (Zeiss).

Supplemental Data

Supplemental Data include two tables and are available with this article online at http://www.developmentalcell.com/cgi/content/full/8/4/587/DC1/.

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